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## Regulation of Autotrophic and Heterotrophic Metabolism in *Pseudomonas oxalaticus* OX1. Growth on Fructose and on Mixtures of Fructose and Formate in Batch and Continuous Cultures

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In *Pseudomonas oxalaticus* the synthesis of enzymes involved in autotrophic CO<sub>2</sub> fixation via the Calvin cycle is regulated by repression/derepression. During growth of the organism on fructose alone, the synthesis of ribulosebiphosphate carboxylase (RuBPCase) remained fully repressed, both in batch culture and in fructose-limited continuous cultures at various dilution rates. Growth in batch culture on a mixture of fructose and formate resulted in the simultaneous utilization of both substrates. Under these conditions we observed synthesis of RuBPCase up to high levels, indicating that formate did not merely function as an ancillary energy source in the metabolism of fructose, but stimulated autotrophic CO<sub>2</sub> fixation via the Calvin cycle. In subsequent experiments growth of *P. oxalaticus* on mixtures of fructose and formate was studied in carbon source-limited continuous cultures. In these experiments further evidence was obtained that fructose is a poor source of (co-)repressor molecules for the synthesis of RuBPCase in the presence of formate. Thus, addition of formate to the medium reservoir of a fructose-limited continuous culture resulted in derepression of RuBPCase synthesis at (relatively) high ratios of fructose over formate. In the reverse experiment the specific activity of RuBPCase decreased with increasing concentrations of fructose in the medium reservoir. However, it can be calculated that the total capacity of RuBPCase in the culture to fix CO<sub>2</sub> remained constant. In these experiments the dry weight produced on the various mixtures equalled the sum of the dry weight values obtained during growth on the same amounts of the two substrates separately. This indicated that, once RuBPCase was present, autotrophic and heterotrophic carbon assimilation pathways functioned simultaneously and independently of each other. Possible explanations for the low repressing effect of fructose on autotrophic CO<sub>2</sub> fixation in *P. oxalaticus* are discussed.

### INTRODUCTION

In the facultatively autotrophic bacterium *Pseudomonas oxalaticus* OX1 synthesis of the enzymes specific for the Calvin cycle, ribulose-1,5-bisphosphate carboxylase (RuBPCase) and phosphoribulokinase, is regulated strongly and in a coordinate manner. During growth on formate, a substrate which is oxidized to carbon dioxide by a soluble (NAD-dependent) and a membrane-bound (NAD-independent) formate dehydrogenase (Quayle, 1961; Dijkhuizen *et al.*, 1980), these enzymes are synthesized up to high levels and carbon assimilation is completely autotrophic (Fig. 1). On the other hand, during growth in batch culture on substrates with a heterotrophic mode of carbon assimilation (e.g. acetate) the activities of these Calvin cycle enzymes are, even when formate is added as a second substrate to the medium, generally not detectable. Although formate is utilized under the latter conditions, it only functions as an

*Abbreviations:* RuBPCase, ribulose-1,5-bisphosphate carboxylase; KDPG aldolase, 2-keto-3-deoxy-6-phosphogluconate aldolase.

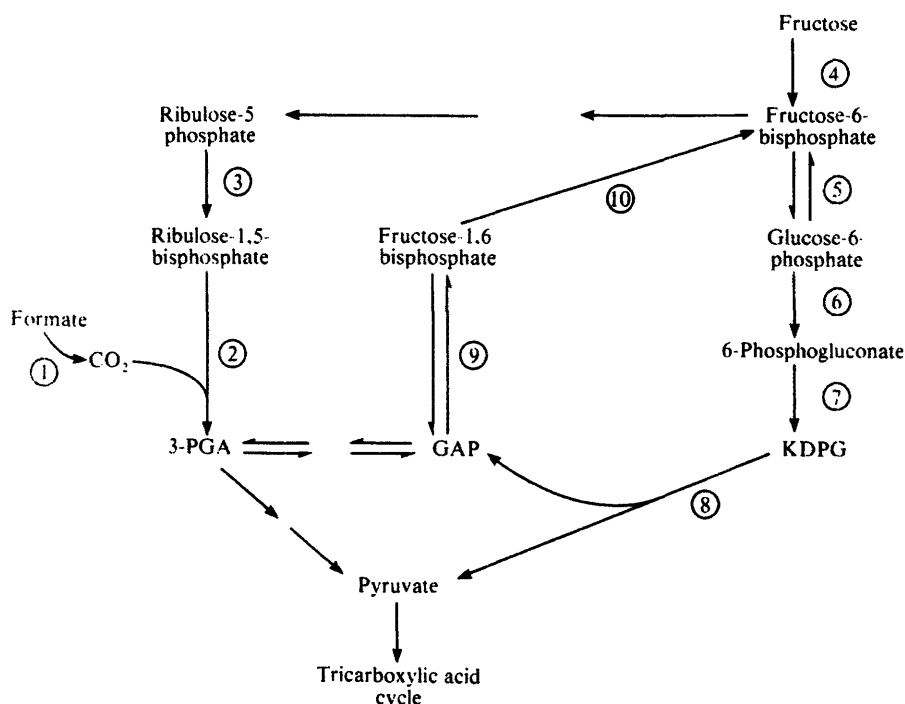


Fig. 1. Metabolism of fructose and formate in *Pseudomonas oxalaticus* OX1. Enzymes: 1, NAD-(in)dependent formate dehydrogenases; 2, ribulose-1,5-bisphosphate carboxylase; 3, phosphoribulokinase; 4, hexokinase; 5, phosphoglucose isomerase; 6, glucose-6-phosphate dehydrogenase; 7, 6-phosphogluconate dehydratase; 8, 2-keto-3-deoxy-6-phosphogluconate aldolase; 9, fructose-1,6-bisphosphate aldolase; 10, fructose-1,6-bisphosphate phosphatase. Abbreviations: 3-PGA, 3-phosphoglycerate; GAP, glyceraldehyde 3-phosphate; KDPG, 2-keto-6-deoxy-6-phosphogluconate.

ancillary energy source, via formate dehydrogenases (Blackmore & Quayle, 1968; Dijkhuizen *et al.*, 1978). A more detailed study of the growth of *P. oxalaticus* on these mixtures (Dijkhuizen & Harder, 1979*a, b*) subsequently showed that in carbon source-limited continuous cultures the repression of the synthesis of the enzymes specific for autotrophic CO<sub>2</sub> fixation generally is less severe. Under these conditions the levels of RuBPCase appeared to depend on the dilution rate employed and on the concentration ratio of the substrates, e.g. formate and acetate, present in the medium reservoir. These studies also showed that the degree of repression observed is strongly dependent on the nature of the 'heterotrophic' substrate involved. Whereas with acetate plus formate repression was severe, growth on oxalate already resulted in derepression of the synthesis of RuBPCase in the complete absence of formate. The latter phenomenon, however, was only observed in chemostat cultures at dilution rates below one-tenth of the  $\mu_{\max}$  on oxalate. From these experiments we concluded that in *P. oxalaticus* the synthesis of the enzymes specific for the Calvin cycle is regulated via a (de)repression mechanism. It was suggested that the degree of repression is probably directly related to the intracellular concentration of the end-product of the Calvin cycle, 3-phosphoglycerate, or a closely related compound (Dijkhuizen & Harder, 1979*a, b*).

Recently, Gordon & McFadden (1980) presented evidence that growth of *P. oxalaticus* in batch culture on fructose alone also resulted in derepression of the synthesis of Calvin cycle enzymes. In view of our earlier studies on the regulation of autotrophic CO<sub>2</sub> fixation in the same organism, this response during growth on fructose is unusual. This substrate, which is metabolized via the Entner–Doudoroff pathway (Gordon & McFadden, 1980; see Fig. 1), therefore seems to represent an exceptional case that merits further investigation. The results of an investigation of metabolic regulation in *P. oxalaticus* during growth on fructose and formate

in batch and continuous cultures are presented in this paper. A preliminary report of this work has been presented elsewhere (Dijkhuizen & Harder, 1983).

## METHODS

*Organism and growth conditions.* *Pseudomonas oxalaticus* OX1 and its maintenance have been described previously (Dijkhuizen & Harder, 1975), as have the experimental conditions of growth on single and mixed substrates in batch (Dijkhuizen *et al.*, 1978) and continuous cultures (Dijkhuizen *et al.*, 1977; Dijkhuizen & Harder, 1979*a, b*).

*Q<sub>O<sub>2</sub></sub> values.* These were determined as described by Dijkhuizen & Harder (1975). The final substrate concentrations used were: formate, 10 mM; fructose, 5 mM. In preliminary experiments it had been shown that these concentrations supported maximal oxygen consumption rates.

*Medium reservoir and residual substrate concentrations.* For the determination of residual substrate concentrations samples were rapidly withdrawn from the culture and filtered through Millipore filters (0.2 µm pore size). Fructose (limit of detection 0.15 mM) was assayed according to Bernt & Bergmeyer (1974) and formate (limit of detection 0.3 mM) as described by Lang & Lang (1972).

*Enzyme assays.* Cell-free extracts were prepared as described by Dijkhuizen *et al.* (1978). Enzyme measurements were made in a Perkin-Elmer type 124 spectrophotometer at 30 °C. In all assay systems the observed rate was linear for at least 3 min and was proportional to the amount of cell-free extract added. Glucose-6-phosphate dehydrogenase (NADP-dependent), EC 1.1.1.49, was assayed according to van Dijken & Quayle (1977). The final reaction mixture (1 ml) contained: potassium phosphate buffer, pH 8.0, 50 µmol; magnesium chloride, 5 µmol; NADP, 0.4 µmol; and bacterial extract. The reaction was started with glucose 6-phosphate, 5 µmol. The combined activities of 6-phosphogluconate dehydratase, EC 4.2.1.12, and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, EC 4.1.2.14, were assayed in the following reaction mixture (1 ml): Tris/HCl buffer, pH 7.5, 50 µmol; reduced glutathione, pH 7.5, 3 µmol; NADH, 0.15 µmol; MnCl<sub>2</sub>, 1 µmol; lactate dehydrogenase, 2 units; and bacterial extract. The reaction was started with 6-phosphogluconate, 5 µmol.

The following enzymes were assayed as described by Dijkhuizen *et al.* (1978): formate dehydrogenase (NAD-dependent), EC 1.2.1.2; formate dehydrogenase (NAD-independent), EC 1.2.-.-; ribulose-1,5-bisphosphate carboxylase, EC 4.1.1.39; phosphoribulokinase, EC 2.7.1.19.

*Carbon dioxide, dry weight and protein determinations.* Carbon dioxide concentrations in culture supernatants and culture dry weight values were determined with a total (organic plus inorganic) carbon analyser (Beckman model 915A), connected to an infrared analyser (Beckman, model 865). Protein in cell-free extracts was determined by the Lowry method using bovine serum albumin as a standard.

*CO<sub>2</sub> fixation by whole cells.* The rate of <sup>14</sup>CO<sub>2</sub> fixation was measured in washed cell-suspensions with excess formate as the energy source (Dijkhuizen *et al.*, 1978).

*Calculations.* The rate of CO<sub>2</sub> fixation required (*R<sub>r</sub>*) to explain the growth rate of *P. oxalaticus* in a formate-limited continuous culture, and the potential rate of CO<sub>2</sub> fixation (*R<sub>p</sub>*), based on the activity of RuBPCase during growth on mixtures of formate and fructose at the various dilution rates, were calculated as described previously (Dijkhuizen & Harder, 1979*a, b*).

## RESULTS

### *Growth on fructose in batch culture*

*Pseudomonas oxalaticus* is able to grow rapidly with fructose as a carbon and energy source. In batch culture, in media containing 5 mM-fructose, we observed a generation time of 2.3 h, close to the value of 2.16 h reported by Gordon & McFadden (1980). These authors presented evidence that, during growth of *P. oxalaticus* in batch culture on fructose alone, RuBPCase and phosphoribulokinase are synthesized to approximately one-third of the levels observed in formate-grown cells. In our experiments we were unable to detect activities of these enzymes, either in the exponential or the stationary growth phases. In this respect fructose behaved as a 'normal substrate' with a heterotrophic mode of carbon assimilation. Growth of *P. oxalaticus* on such substrates in batch cultures, in the absence of formate, has so far invariably resulted in total repression of autotrophic CO<sub>2</sub> fixation (Blackmore & Quayle, 1968; Dijkhuizen *et al.*, 1978).

### *Growth on a mixture of fructose and formate in batch culture*

The effectiveness of a particular substrate in acting as a source of (co-)repressor molecule(s) for the synthesis of RuBPCase in *P. oxalaticus* manifests itself most clearly during growth of the organism on a mixture of formate and the substrate (Dijkhuizen *et al.*, 1978). The pattern of

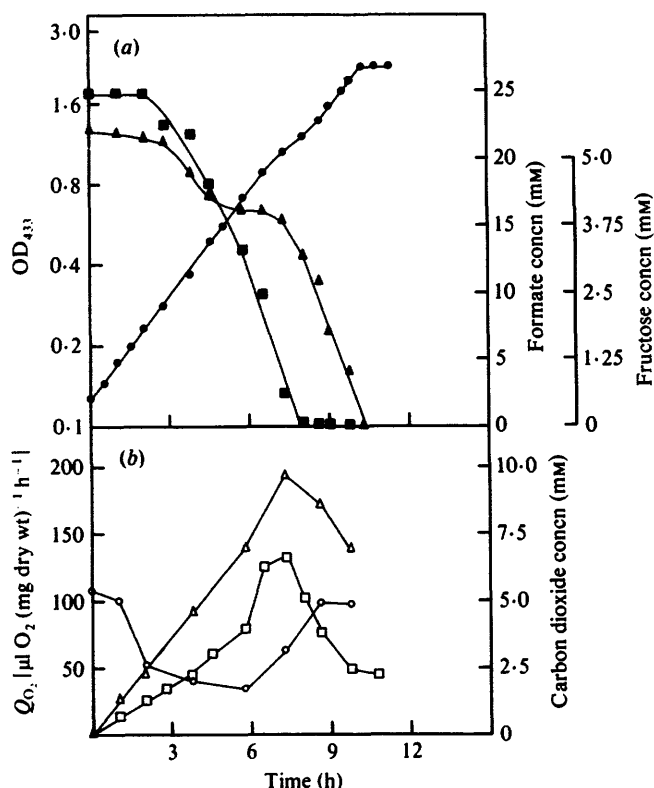


Fig. 2. Growth of *Pseudomonas oxalaticus* OX1 on a mixture of fructose (5 mM) plus formate (25 mM) in batch culture. The inoculum was pregrown on fructose (5 mM). (a) Optical density (●), fructose concentration (▲), formate concentration (■). (b)  $Q_{O_2}$ -formate (Δ),  $Q_{O_2}$ -fructose (○), carbon dioxide concentration (□).

growth of *P. oxalaticus*, pregrown on fructose, and inoculated into a mixture of fructose (5 mM) and formate (25 mM) in batch culture was rather complex (Fig. 2). Growth on fructose started immediately (Fig. 2a), but also the capacity of washed cell-suspensions to oxidize formate appeared rapidly (Fig. 2b). From 2 h and onwards after the start of the experiment this clearly resulted in the simultaneous utilization of both substrates. Throughout the experiment the generation time characteristic for growth on fructose (2.3 h) was maintained, except for a short period after the exhaustion of formate (7–9 h). After 5 h the rate of fructose utilization slowed down markedly, but recovered again after the exhaustion of formate (Fig. 2a). This pattern of substrate utilization was also reflected in the capacity of washed cell-suspensions to oxidize fructose and formate and by the accumulation of CO<sub>2</sub> (Fig. 2b). Thus, the  $Q_{O_2}$ -fructose passed through a minimum whereas the amounts of CO<sub>2</sub> produced and the  $Q_{O_2}$ -formate increased until the exhaustion of formate from the medium. The activities of enzymes characteristic for growth on formate and fructose (see Fig. 1) are summarized in Table 1. The activities of NAD-dependent formate dehydrogenase and RuBPCase, absent in cells pregrown on fructose, increased rapidly in the first 5–7 h (during growth with formate) and decreased again after the exhaustion of this substrate. In the first 6–7 h, glucose-6-phosphate dehydrogenase and the Entner–Doudoroff pathway enzymes, 6-phosphogluconate dehydratase and KDPG aldolase, however, lost about 50% of their activities present in cultures pregrown on fructose. In this period the bacterial mass had multiplied approximately three times, indicating that synthesis of these enzymes had not totally stopped but had become partly repressed. Taken together, these results show that growth of *P. oxalaticus* in batch culture on a mixture of fructose and formate

Table 1. Enzyme activities [ $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ ] during growth of *Pseudomonas oxalaticus* OX1, pregrown on fructose, on 5 mM-fructose, on 5 mM-fructose plus 25 mM-formate

Time (h)	RuBPCase	NAD-formate dehydrogenase	Glucose-6-phosphate dehydrogenase	Entner-Doudoroff enzyme system*
0	0	0	103	122
1	14	54	85	97
2	17	58	71	89
3.45	26	134	67	60
5.45	58	189	50	45
7.15	113	60	57	41
8.36	50	26	107	59
10.45	18	6	129	92

\* Combined activities of 6-phosphogluconate dehydratase and KDPG aldolase.

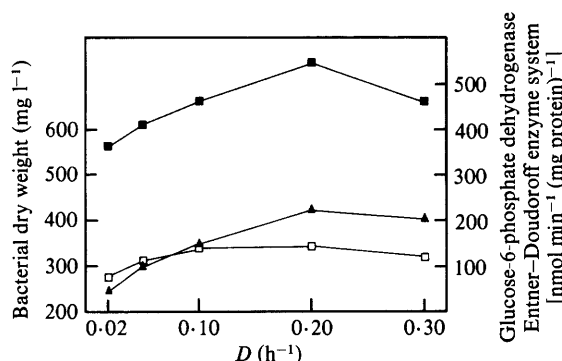


Fig. 3. Growth of *Pseudomonas oxalaticus* OX1 in a fructose-limited ( $S_R = 5$  mM) continuous culture.  $\blacktriangle$ , Bacterial dry weight;  $\blacksquare$ , glucose-6-phosphate dehydrogenase;  $\square$ , Entner-Doudoroff enzyme system.

results both in the utilization of formate as an energy source and in autotrophic  $\text{CO}_2$  fixation. The results therefore also indicate that under these conditions fructose, compared to acetate and glycollate (Dijkhuizen *et al.*, 1978), is a weak source of (co-)repressor molecule(s) for the synthesis of Calvin cycle enzymes.

#### Growth on fructose in continuous culture

Previous studies (Dijkhuizen & Harder, 1979*a, b*) showed that during growth of *P. oxalaticus* on mixtures of oxalate or acetate plus formate, in carbon source-limited continuous cultures, the repression of autotrophic  $\text{CO}_2$ -fixing enzymes is less severe as compared to the situation in batch cultures. However, only with one (single) substrate, namely oxalate (Dijkhuizen & Harder, 1979*b*), have we so far observed synthesis of RuBPCase at relatively low dilution rates in continuous culture. In view of the results described above we decided to study growth of *P. oxalaticus* in carbon source-limited continuous cultures on fructose alone and on mixtures of fructose plus formate. In these experiments the various measurements were made after the cultures had reached a steady-state.

When *P. oxalaticus* was grown in a fructose-limited continuous culture ( $S_R$  fructose = 5 mM), the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase/KDPG aldolase increased with increasing dilution rate, reached a maximum at  $D = 0.20 \text{ h}^{-1}$  and decreased slightly as the dilution rate was further increased to  $0.30 \text{ h}^{-1}$  (Fig. 3). No residual fructose was detectable in supernatants of these cultures. The bacterial dry weight values measured during growth at the various dilution rates are shown in Fig. 3. Not

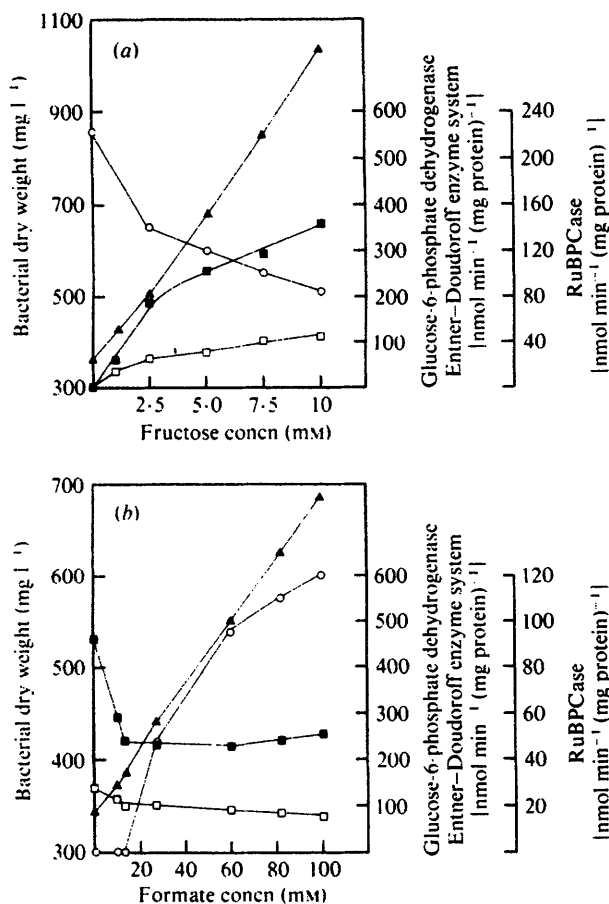


Fig. 4. (a) Effect of increasing concentrations of fructose (0–10 mM) in the reservoir of a formate-limited ( $S_R = 100 \text{ mM}$ ) continuous culture of *Pseudomonas oxalaticus* OX1 at  $D = 0.10 \text{ h}^{-1}$  on a number of steady-state culture parameters. (b) Effect of increasing concentrations of formate (0–100 mM) in the reservoir of a fructose-limited ( $S_R = 5 \text{ mM}$ ) continuous culture of *Pseudomonas oxalaticus* OX1 at  $D = 0.10 \text{ h}^{-1}$  on a number of steady-state culture parameters. ▲, Bacterial dry weight; ■, glucose-6-phosphate dehydrogenase; □, Entner–Doudoroff enzyme system; ○, RuBPCase.

surprisingly, the molar growth yield of the organism, calculated at, for instance,  $D = 0.10 \text{ h}^{-1}$ , is much higher on fructose ( $70 \text{ g mol}^{-1}$ ) than on formate ( $3.6 \text{ g mol}^{-1}$ ; Dijkhuizen & Harder, 1979*a, b*). In these experiments no activity of formate dehydrogenases, RuBPCase or phosphoribulokinase was detected, as was the case in batch culture during growth on fructose alone. This indicates that, even under carbon- and/or energy-limiting conditions, the repressive effect of fructose metabolism is strong enough to prevent synthesis of the Calvin cycle enzymes (at dilution rates between  $0.02$  and  $0.30 \text{ h}^{-1}$ ).

#### Growth on mixtures of fructose and formate in continuous culture

The effect of increasing concentrations of fructose in the medium reservoir of a formate-limited continuous culture ( $S_R$  formate =  $100 \text{ mM}$ ) of *P. oxalaticus*, growing at a constant dilution rate of  $0.10 \text{ h}^{-1}$ , is shown in Fig. 4(a). At all ratios of fructose and formate in the feed, residual substrate in the culture was not detected. With increasing amounts of fructose in the medium reservoir a progressive increase in the activities of enzymes of fructose metabolism was observed. At a fructose concentration of  $5 \text{ mM}$ , the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase/KDPG aldolase (Fig. 4a) were approxi-

Table 2. Capacity of RuBPCase to fix CO<sub>2</sub> at various concentrations of fructose in the reservoir of a formate-limited culture of *Pseudomonas oxalaticus* OX1 at  $D = 0.10 \text{ h}^{-1}$ 

Fructose concn (mM)	0	2.5	5	7.5	10
$R_p$ : potential rate of CO <sub>2</sub> fixation	2.52	2.22	2.58	2.70	2.73
$R_r$ : required rate of CO <sub>2</sub> fixation*	1.43	1.43	1.43	1.43	1.43
$R_p/R_r$ : capacity of RuBPCase	1.76	1.55	1.80	1.89	1.91

\* Calculated on the assumption that the same amount of cell material was synthesized from CO<sub>2</sub> during growth on the mixture as during growth of the organism on formate alone.

mately 50% of the levels of these enzymes observed during growth on fructose alone at the same dilution rate (see Fig. 3). However, a comparison of the bacterial dry weight values obtained under these growth conditions (675 and 359 mg l<sup>-1</sup>, respectively) showed that per litre these cultures apparently had a similar potential to metabolize fructose. As the fructose concentration in the medium reservoir was increased, the specific activities of both formate dehydrogenases remained fairly constant (data not shown). The synthesis of RuBPCase, however, became progressively repressed (Fig. 4a), and the rate of <sup>14</sup>CO<sub>2</sub> fixation by whole cells decreased similarly (data not shown). A calculation of the specific activity of RuBPCase required to synthesize the same amount of cell material from CO<sub>2</sub> during growth on the mixture as during growth on formate alone not only showed that the remaining activity of RuBPCase was still sufficiently high to fulfil this task (Table 2), but also that the capacity of RuBPCase remained fairly constant. Since the amount of bacterial dry weight produced on the mixture also almost equalled the sum of the dry weight values obtained during growth on fructose and formate separately, we conclude that under these conditions both heterotrophic and autotrophic carbon assimilation pathways functioned simultaneously and independently of each other. Additive growth yields were also obtained in experiments in which oxalate or acetate were added to the reservoir of a formate-limited culture (Dijkhuizen & Harder, 1979a, b).

The results obtained in the reverse experiment, in which the effect of increasing formate concentrations in the reservoir of a fructose-limited continuous culture ( $S_R$  fructose = 5 mM) of *P. oxalaticus* was studied at a dilution rate of  $0.10 \text{ h}^{-1}$ , are shown in Fig. 4(b). Again, at all ratios of fructose and formate in the feed, residual substrate was not detected in the culture. Addition of formate to the medium reservoir immediately resulted in synthesis of both formate dehydrogenases, and high specific activities of these enzymes were observed at the higher formate concentrations (data not shown). Concomitantly, the capacity of washed suspensions to oxidize formate appeared and increased, in a linear fashion, up to a (high) level comparable to that observed during growth on 100 mM-formate alone (Dijkhuizen & Harder, 1979b) at the same dilution rate [741 and 618  $\mu\text{l O}_2 (\text{mg dry wt})^{-1} \text{ h}^{-1}$ , respectively]. With 10–13 mM-formate no activity of RuBPCase was detectable, although formate was oxidized completely to CO<sub>2</sub> by way of the formate dehydrogenases. This utilization of formate as an ancillary energy source resulted in an increase (13%) in bacterial dry weight of the culture. Since fructose was the only available carbon source this must have been achieved by a slight increase in the amount of fructose used for the synthesis of cell material. In this range of formate concentrations the specific activities of Entner–Doudoroff pathway enzymes decreased. At concentrations of formate in the feed above 13 mM, synthesis of RuBPCase did occur and the specific activity of this enzyme increased steeply. With 100 mM-formate, RuBPCase reached the same specific activity as observed in the reverse experiment (see above) with 5 mM-fructose. The same pattern was observed in measurements of the rate of <sup>14</sup>CO<sub>2</sub> fixation by whole cells (data not shown). A calculation of the ratio of the potential rate of CO<sub>2</sub> fixation ( $R_p$ ) and the required rate of CO<sub>2</sub> fixation ( $R_r$ ) of the culture showed (Table 3) that this parameter was immediately above unity once RuBPCase synthesis occurred. In this experiment again, the amount of bacterial dry weight produced on the mixture almost equalled the sum of the dry weight values obtained during growth on fructose and formate separately. These data therefore indicate that, once RuBPCase synthesis occurred, the rate of CO<sub>2</sub> fixation by the cells was independent of the presence of fructose.



Table 3. Capacity of RuBPCase to fix  $\text{CO}_2$  at various concentrations of formate in the reservoir of a fructose-limited culture of *Pseudomonas oxalaticus* OX1 at  $D = 0.10 \text{ h}^{-1}$

Formate concn (mM) . . .	13	27	60	82	100
$R_p$ : potential rate of $\text{CO}_2$ fixation	—	0.67	1.66	2.16	2.58
$R_r$ : required rate of $\text{CO}_2$ fixation*	0.19	0.39	0.86	1.17	1.43
$R_p/R_r$ : capacity of RuBPCase	—	1.72	1.93	1.85	1.80

\* Calculated on the assumption that the same amount of cell material was synthesized from  $\text{CO}_2$  during growth on the mixture as during growth of the organism on formate alone.

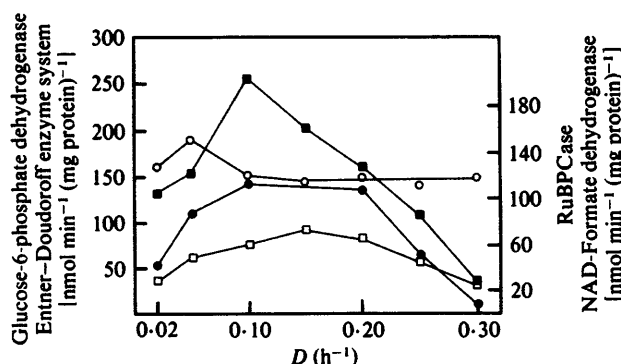


Fig. 5. Relationship between enzyme activity and dilution rate during growth of *Pseudomonas oxalaticus* OX1 on a constant mixture of fructose ( $S_R = 5 \text{ mM}$ ) and formate ( $S_R = 100 \text{ mM}$ ). ■, Glucose-6-phosphate dehydrogenase; □, Entner-Doudoroff enzyme system; ○, RuBPCase; ●, NAD-formate dehydrogenase.

In previous experiments (Dijkhuizen & Harder, 1979*a, b*), in which the effect of increasing amounts of formate in the reservoir of an oxalate- or acetate-limited continuous culture of *P. oxalaticus*, at  $D = 0.10 \text{ h}^{-1}$ , was studied, RuBPCase synthesis was only observed at formate concentrations of 43 or 50 mM, respectively, and higher. Below these concentrations the energy derived from formate oxidation only served to increase the synthesis of cell material from oxalate- or acetate-carbon. The results obtained in the experiment described in this section therefore indicate that fructose is only a weak source of repressor molecule(s) for the synthesis of RuBPCase under these conditions.

During growth of *P. oxalaticus* in a carbon and energy-limited continuous culture with a constant mixture of 5 mM-fructose and 100 mM-formate in the medium reservoir, at various dilution rates, both the level of RuBPCase (Fig. 5) and the capacity of whole cells to fix  $^{14}\text{CO}_2$  (data not shown) remained fairly constant. At dilution rates below  $0.25 \text{ h}^{-1}$  the specific activity of RuBPCase was sufficiently high to allow the cells to assimilate  $\text{CO}_2$  via the Calvin cycle as if fructose were not present in the mixture ( $R_p/R_r$  values above 1; Table 4). The growth yield data indicate (Fig. 6) that this was actually the case, since the dry weight of organisms produced on the mixture was almost exactly the same as the sum of the dry weights found during growth on the single substrates. At dilution rates of  $0.25$  and  $0.30 \text{ h}^{-1}$  the RuBPCase activities were too low to accomplish this (Table 4) but the growth yields remained additive. At  $D$  values above  $0.20 \text{ h}^{-1}$ , the maximum growth rate of *P. oxalaticus* on formate alone, the specific activities of the formate dehydrogenases, represented in Fig. 5 by the NAD-formate dehydrogenase, progressively decreased during growth on the mixture. In addition above  $D = 0.10$ – $0.15 \text{ h}^{-1}$ , an increasing repression of the synthesis of Entner-Doudoroff pathway enzymes was observed during growth on the mixture. At dilution rates above  $0.15 \text{ h}^{-1}$  care had to be taken that the rate of medium supply to the culture vessel was increased relatively slowly. In early experiments, in which the higher dilution rates were established at  $0.05 \text{ h}^{-1}$  intervals, we observed

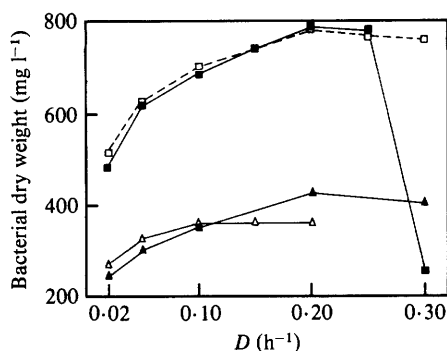


Fig. 6. Relationship between bacterial dry weight and dilution rate during growth of *Pseudomonas oxalaticus* OX1 on: formate ( $S_R = 100$  mM) ( $\Delta$ ), fructose ( $S_R = 5$  mM) ( $\blacktriangle$ ), mixture of formate ( $S_R = 100$  mM) and fructose ( $S_R = 5$  mM) ( $\blacksquare$ ).  $\square$ , Sum of the bacterial dry weight values obtained during growth on formate and fructose separately; the values at  $D = 0.25$  and  $0.30$  h $^{-1}$  for growth on formate alone were obtained by extrapolation.

Table 4. Capacity of RuBPCase to fix  $CO_2$  during growth of *Pseudomonas oxalaticus* OX1 on a mixture of fructose (5 mM) and formate (100 mM) in the reservoir of a carbon source-limited culture at different dilution rates

Dilution rate (h $^{-1}$ )	0.02	0.05	0.10	0.15	0.20	0.25	0.30
$R_p$ : potential rate of $CO_2$ fixation	1.92	2.94	2.58	2.69	2.94	2.74	0.95
$R_r$ : required rate of $CO_2$ fixation*	0.21	0.66	1.43	2.15	2.86	3.58	4.28
$R_p/R_r$ : capacity of RuBPCase	9.14	4.46	1.80	1.25	1.03	0.77	0.22

\* The required rate of  $CO_2$  fixation was calculated on the assumption that the same amount of cell material was synthesized from  $CO_2$  during growth on the mixture as during growth of the organism on formate alone.

accumulation of residual substrates up to various concentrations resulting in unstable cultures for prolonged periods. This was probably due to the growth-inhibitory effects of formate (Dijkhuizen & Harder, 1975). When the dilution rate was increased at 0.02 h $^{-1}$  intervals, only at  $D = 0.30$  h $^{-1}$  (close to the  $\mu_{max}$  on fructose alone) did part of the fructose and formate (3.8 and 46 mM, respectively) remain unutilized.

## DISCUSSION

In a study of fructose metabolism in *P. oxalaticus*, Gordon & McFadden (1980) showed that growth of the organism in batch culture on this substrate alone resulted in synthesis of the enzymes specific for the Calvin cycle. Our experiments, both in batch culture and fructose-limited continuous culture, show that this is not an inevitable consequence of growth of the organism on fructose. In facultatively autotrophic bacteria derepression of the synthesis of the key enzymes of the Calvin cycle during growth on substrates that sustain a heterotrophic mode of carbon assimilation is not an unusual phenomenon. This has, for instance, been observed in various members of the *Rhodospirillaceae*, when they are grown aerobically in the dark (Lascelles, 1960), and in the hydrogen bacterium *Alcaligenes eutrophus* H16 (Friedrich *et al.*, 1981). In *A. eutrophus* this is the case during growth in batch culture on the (single) organic compounds fructose, gluconate or glycerol. Friedrich *et al.* (1981) also observed that during growth of *A. eutrophus* in batch cultures on succinate the (total) repression of the synthesis of the Calvin cycle enzymes which is normally exerted by this substrate can be overcome (partly) by employing environmental conditions which result in oxygen depletion, or by maintaining the pH of the culture at a value which is suboptimal for growth. Although it is not directly evident from the methodology employed by Gordon & McFadden (1980), one might speculate that

differences in one or more of these growth parameters underly the contradictory results obtained in studies on metabolic regulation in *P. oxalaticus*.

Nevertheless, the results described in this paper provide substantial support for the conclusion that, compared with, for instance, acetate, fructose is a poor source of (co-)repressor molecules for the synthesis of RuBPCase during growth of *P. oxalaticus* on mixtures of fructose plus formate. In contrast to the situation with acetate (Dijkhuizen & Harder, 1979*a*) addition of fructose to the medium reservoir of a formate-limited continuous culture did not result in a decreased capacity of RuBPCase in the culture to fix CO<sub>2</sub>, and in the reverse experiment derepression of RuBPCase synthesis was observed already at (relatively) high ratios of fructose over formate in the medium reservoir. Finally, growth of the organism on the mixture in batch culture resulted in the synthesis of RuBPCase up to high levels, a phenomenon which, again, did not occur with acetate (Dijkhuizen *et al.*, 1978). In the present study comparable results were obtained during growth of *P. oxalaticus* on mixtures of either fructose or gluconate plus formate.

We previously postulated that in *P. oxalaticus*, and probably in autotrophic bacteria in general, the degree of repression of the synthesis of the two enzymes specific for the Calvin cycle is determined by the intracellular concentration of 3-phosphoglycerate or closely related metabolites (Knight *et al.*, 1978). The results obtained during growth on fructose plus formate are not in disagreement with this hypothesis. First of all, it is quite possible that during growth on fructose alone the rate-limiting step in the metabolism of this compound is in one of the enzymes of the Entner-Doudoroff pathway (see Fig. 1). Secondly, our experiments show that the utilization of fructose becomes impaired in the presence of formate, both in batch culture and at the higher *D* values in continuous culture. This effect of formate was not studied in further detail, but may be comparable to the so-called hydrogen effect in *A. eutrophus*, in which the activity and synthesis of glucose-6-phosphate dehydrogenase is inhibited in the presence of hydrogen, a source of NADH<sub>2</sub> and ATP (Bowien *et al.*, 1974). Thirdly, in earlier experiments we showed that addition of formate to the reservoir of an acetate-limited continuous culture of *P. oxalaticus* resulted in an increase in the proportion of acetate-carbon assimilated via the glyoxylate cycle, and a decrease in the amount of acetate dissimilated via the tricarboxylic acid cycle. In these experiments we also observed that the redistribution of acetate-carbon in the presence of formate as an ancillary energy source, i.e. in the absence of RuBPCase or at *R<sub>p</sub>/R<sub>t</sub>* values below unity, resulted in an increase in the amount of dry weight produced on the mixture, compared with the sum of the dry weight values obtained during growth on the same amounts of these substrates separately. This phenomenon, which is a reflection of the energy-saving effect of fixing less CO<sub>2</sub> via the energetically expensive Calvin cycle (Dijkhuizen & Harder, 1979*a, b*), has also been observed in *Thiobacillus* A2 during growth on mixtures of thiosulphate and acetate (Gottschal & Kuenen, 1980). In the present study of growth of *P. oxalaticus* on fructose plus formate, however, this phenomenon was not observed, either at formate concentrations of 13 mM and lower (at *D* = 0.10 h<sup>-1</sup>; Fig. 4*b*) nor at *D* = 0.25 h<sup>-1</sup> (on 5 mM-fructose plus 100 mM-formate; Fig. 6). By comparison, growth of *Thiobacillus* A2 on mixtures of glucose plus thiosulphate (Smith *et al.*, 1980) and glucose plus formate (Wood & Kelly, 1981), or growth of *Thiobacillus novellus* (Leefeldt & Matin, 1980) on mixtures of glucose plus thiosulphate, resulted in the production of amounts of bacterial dry mass which were exactly the sum of those obtained on the single substrates. Conceivably, upon addition of formate (or thiosulphate) such a redistribution of the flow of carbon from a heterotrophic substrate (e.g. acetate) might result in a continued production of metabolites such as 3-phosphoglycerate, and therefore in a prolonged repression of the synthesis of RuBPCase. Addition of formate to the medium reservoir of a fructose-limited continuous culture of *P. oxalaticus* apparently did not result in a major redistribution of fructose carbon, and RuBPCase synthesis started at relatively low formate concentrations. A major redistribution of fructose carbon, conceivably resulting in a prolonged repression of RuBPCase synthesis, is also unlikely to occur because the main flow of fructose carbon is via metabolites such as 3-phosphoglycerate anyway, both for energy generation and carbon assimilation.

In conclusion, growth of *P. oxalaticus* on fructose plus formate apparently results in a reduction in the intracellular pool of (co-)repressor molecules for RuBPCase synthesis. A physiological explanation for this may be found in either of the three situations outlined above.

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